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Molecular Cloning and Expression of *ptxA*, the Gene Encoding the 120-Kilodalton Cytotoxin of *Actinobacillus pleuropneumoniae* Serotype 2

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The genetic determinants of the 120-kDa cytotoxin of *Actinobacillus pleuropneumoniae* serotype 2 were isolated from a lambda DNA library by a plaque immunoblot technique. Expression of the 120-kDa polypeptide was confirmed by Western immunoblot analysis of infected *Escherichia coli* cell lysates, which were shown to be toxic for porcine alveolar macrophages in vitro. The genetic determinants of the toxin were subcloned into the plasmid vector pUC18. This plasmid (pPTX1) directed the synthesis and secretion of the active 120-kDa cytotoxin in *E. coli*. The recombinant toxin was indistinguishable from native cytotoxin from *A. pleuropneumoniae* serotype 2 with respect to molecular size, reaction in Western blot analysis, heat lability, cytotoxic activity, and neutralization by serum antibody. A restriction endonuclease cleavage map of pPTX1 was prepared, and deletion mutants were used to locate the minimal region of DNA required for production of intracellular toxin; this gene was termed *ptxA*. Southern hybridization analysis with a 1.7-kb *Pvu*II fragment located within the *ptxA* gene revealed sequences with a high degree of homology in serotype reference strains 2, 3, 4, 6, and 8. Other reference strains did not contain sequences that were recognized by this probe. However, related sequences (>71% homology) were detected in *Actinobacillus actinomycetemcomitans* and *A. equuli*. Weak hybridization was observed between the *ptxA* probe and pLKT5, which carries the *lktAC* genes of *Pasteurella haemolytica*, and between the *ptxA* probe and pAPH1, which carries the structural gene for type II hemolysin from *A. pleuropneumoniae*. The isolation of the genetic determinants of this cytotoxin will enable investigations of the structure and organization of the *ptx* DNA region and further analysis of its role in the pathogenesis of pleuropneumonia.

Contagious porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is of major importance in intensive pig production (34). Acute infection usually takes the form of a fibrinous pleurisy and widespread hemorrhagic pneumonia (22). In the chronic form of the disease, which is usually found in animals with some degree of immunity, localized lesions of pulmonary necrosis and fibrous adhesion to the pleura are found. The pathogenesis of the disease is consistent with the production by the bacteria of one or more toxins (2, 3, 18). In support of this was the finding that typical lesions of pleuropneumonia could be induced by endobroncheal administration of sonicated bacteria or sterile culture supernatant (30). Toxic activity for cells in vitro was first reported by Bendixen et al. (1). A heat-stable substance produced by serotype 2 strains was associated with toxicity for porcine alveolar macrophages (17). Other investigators have shown the existence of a heat-labile neutrophil toxin secreted by a number of different serotypes (15, 29, 37).

The cytotoxic activity of *A. pleuropneumoniae* was considered to be caused by the protein that caused the hemolytic activity (17, 29). The hemolysin of serotype 1 (HlyI) has been purified and shown to be a polypeptide of approximately 105 to 110 kDa (7, 9). This was supported by the cloning of the genes encoding the hemolysin from serotype 5 (4) and from serotype 1 (10). Furthermore, the hemolysin was shown to be related to the RTX family of bacterial protein toxins (4, 5, 10, 35). A second hemolysin (HlyII), found in a serotype 2 strain, was distinguished on the basis of its requirement for calcium ions for its activity (8). HlyII has been partially characterized and found to be different from HlyI but immunologically related (8). However, no direct evidence showing that the hemolytic and cytotoxic activities are caused by the same substance was presented.

Studies on the production of hemolysin and cytotoxin among *A. pleuropneumoniae* isolates suggested that the two activities could vary independently among strains (12, 29). Further genetic studies demonstrated the two activities to be distinct and that the cytotoxin, termed pleurotoxin (PTX), is a 120-kDa polypeptide produced by a majority of strains among serogroups 2, 3, 4, and 5 (32).

Little is known of the possible role of this cytotoxin in the pathogenesis of pleuropneumonia. However, it has a rapidly lethal effect on porcine alveolar macrophages and porcine neutrophils in vitro (29, 32, 37) and might severely impair the nonspecific cellular defenses of the pig lung during infection. To allow further study of the toxin and the genes determining its production, we cloned the gene for PTX in *Escherichia coli*.

In this paper we describe the isolation and preliminary characterization of the gene for PTX from *A. pleuropneumoniae* serotype 2. We have termed the gene *ptxA*. In addition, we demonstrate the production and export of the 120-kDa polypeptide in *E. coli* and compare it functionally with the native protein. Finally, we examine the distribution of *ptxA*-related genes among other serotypes of *A. pleuropneumoniae* and other members of the genus *Actinobacillus*.

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TABLE 1. Bacterial strains

Bacterial strain	Serotype	Source or reference(s)
A. pleuropneumoniae		
HK361	2	16
HK361e	2	32
4074	1	16
S1536	2	16
S1421	3	16
M62	4	16
K17	5a	16, 25
L20	5b	16, 25
Femø	6	28
WF83	7	28
405	8	27
CVI13261	9	23
13039	10	24
56153	11	14
8329	12	26
E. coli		
JM109		39
DH1		11
A. lignieresii		This laboratory
A. suis		This laboratory
A. actinomycetemcomitans JP2		36
A. equuli		This laboratory

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. Strains of *A. pleuropneumoniae* and other bacteria used in the study are listed in Table 1. Bacteriophage lambda cloning vector EMBL3 and its host *E. coli* KW251 were obtained from Promega Corp., Madison, Wis. Plasmid vector pUC18 (39) was used in all of the subcloning experiments with *E. coli* JM109 as the host. Plasmid pLKT5, carrying the *lktCA* genes (35), was donated by John Coote. Plasmid pAPH1, carrying the structural gene for the type II hemolysin of *A. pleuropneumoniae* serotype 2, was constructed in this laboratory (unpublished data). *E. coli* DH1 (11) was used in studies of expression and toxin secretion.

Culture conditions. *A. pleuropneumoniae* strains were grown as previously described (32). *E. coli* strains were grown in L broth or L agar (19) supplemented when necessary with ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-β-D-thiogalactopyranoside for selection of recombinant plasmids in JM109.

Preparation and screening of the DNA library. High-molecular-weight genomic DNA was prepared from 100-ml stationary-phase cultures of *A. pleuropneumoniae* HK361, serotype 2 (19). The strain is cytotoxic and produces calcium-dependent (type II) hemolytic activity (16, 32). Bacterial DNA was partially digested with *Sau*3A and size fractionated on a 10 to 40% (wt/vol) sucrose gradient (19). Fractions containing fragments of DNA in the size range of 9 to 23 kb were pooled, mixed with lambda EMBL3 arms at a molar ratio of 1:1, and treated with T4 DNA ligase for 4 h at 23°C. The ligated DNA was packaged in vitro (19) and plated on *E. coli* KW251 for screening without amplification.

Plaques were transferred to nitrocellulose filters and blocked in 10 mM Tris (pH 8.0)–150 mM NaCl–0.05% Tween 20 containing 10% skimmed milk powder for 15 min. Filters were incubated overnight at 4°C with convalescent-phase pig serum (1:500 in blocking solution). To ensure that serotype-specific antigens were not detected in the plaques, the serum used was taken from an animal after infection with a heterologous (serotype 3) strain of *A. pleuropneumoniae* (31). The background of nonspecific reaction with the plaques was removed by absorbing the serum with a sonic lysate of the lambda host strain *E. coli* KW251 (10^{10} bacteria per ml of serum). Reactive plaques were visualized with rabbit antipig immunoglobulin-horseradish peroxidase (1:2,000; Sigma) and a substrate for 1 h as previously described (32).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Bacteriophage lysates and culture supernatants were analyzed as previously described (32). Murine monoclonal antibody VPG14, which specifically recognizes the 109-kDa HlyII hemolysin, was isolated in this laboratory (unpublished data). VPG14 was diluted 1:20 for use as a probe in immunoblotting. Polyclonal antibody against the 120-kDa cytotoxin was raised in a rabbit against PTX purified by preparative gel electrophoresis of culture supernatant from the hemolysin-deficient mutant HK361e. This was diluted 1:500 for immunoblotting.

Subcloning and transformation procedures. Restriction endonucleases were purchased from Life Tech, Paisley, Scotland, and used according to the manufacturer's recommendations. Plasmid DNA was prepared by the method of Clewell and Helinski (6). Deletion mutants were constructed by ligation of fragments of the digested recombinant plasmid pPTX1, or its derivatives, into digested vector pUC18. Fragments of DNA for subcloning were purified by agarose electrophoresis, passed through a Spin-X column (Costar, Cambridge, Mass.), and extracted by the Gene Clean procedure (Bio-101, La Jolla, Calif.). Bacterial transformation was performed by the method of Hanahan (11).

DNA hybridization procedures. Probe DNA was extracted from preparative agarose gels with Gene Clean and labeled with random hexadeoxynucleotide primers (Pharmacia oligolabeling kit) and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham).

Total bacterial DNA or plasmid DNA was digested to completion with the appropriate restriction enzyme, and fragments were separated in 0.6% agarose before transfer to nitrocellulose filters (Bio-Rad). Prehybridization was for 4 h at 55 or 68°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.1% SDS (19). Hybridization was carried out overnight at 55°C (low stringency) or 68°C (high stringency) in the same solution, with the addition of 100 μg of denatured salmon sperm DNA (Sigma) per ml and 25 ng of denatured probe DNA. Filters were washed at 25°C in 2× SSC–1× Denhardt's solution–0.1% SDS twice for 30 min and at 55°C in 1.0× SSC–0.1% SDS (low stringency) or at 68°C in 0.1× SSC–0.1% SDS (high stringency) twice for 1 h. Hybridizing DNA was visualized by autoradiography at −70°C.

Cytotoxic activity, cytotoxin-neutralizing antibody, and hemolysin activity. The cytotoxicity of preparations was assessed as previously described (32). Briefly, 100 μl of test material was added to a well containing 10^6 fresh porcine alveolar macrophages in 900 μl in minimal essential medium and incubated for 60 min at 37°C in an atmosphere of 3% CO_2 in air. Toxicity was determined microscopically by the extent of cellular damage (loss of refractility) and uptake of Trypan blue dye. One unit of cytotoxic activity was defined as the quantity of toxin that induced 50% of the cells to stain with 0.02% trypan blue or to become detached from the

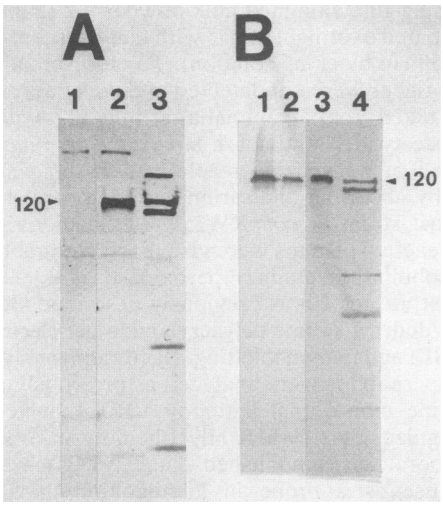

FIG. 1. Expression of *A. pleuropneumoniae* 120-kDa antigen in *E. coli*, determined by Western immunoblotting. (A) Lanes: 1, *E. coli* KW251 bacteriophage lysate from recombinant EMBL3 carrying *A. pleuropneumoniae* DNA, which did not react with convalescent-phase serum in the plaque immunoblot; 2, *E. coli* KW251 lysate from recombinant EMBL3 clone 13; 3, culture supernatant from *A. pleuropneumoniae* HK361 (control). (B) Lanes: 1, cell lysate from *E. coli* JM109(pPTX1); 2, JM109(pPTX2) carrying the same cloned insert in the alternative orientation; 3, cell-free culture supernatant from *E. coli* DH1(pPTX1); 4, *A. pleuropneumoniae* HK361 culture supernatant (control). Filters were probed with convalescent-phase pig serum absorbed with *E. coli* lysate as described in Materials and Methods.

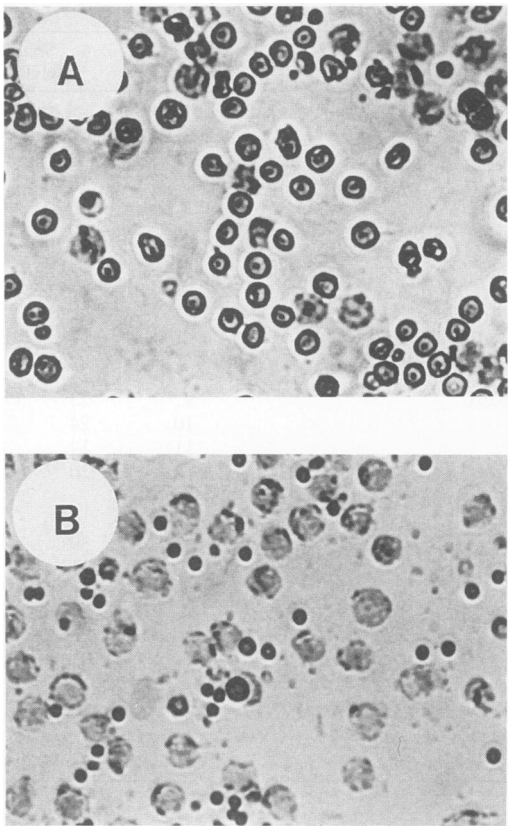

FIG. 2. Toxicity of rPTX. (A) Porcine alveolar macrophages treated for 1 h with 10% culture supernatant from *E. coli* DH1 pUC18; (B) porcine alveolar macrophages treated for 1 h with 10% culture supernatant from *E. coli* DH1(pPTX1) after 6 h of growth.

culture plate, in a volume of 1.0 ml, after incubation for 60 min at 37°C. Cytotoxin-neutralizing antibody was measured by mixing 50 μl of active cytotoxin (80 U/ml) with an equal volume of serum or serum diluted in 150 mM NaCl at 0°C for 60 min. The sample was then assayed for cytotoxic activity as previously described, and the neutralizing titer was expressed as the reciprocal dilution of serum required to entirely prevent the cytotoxic effect of the test sample. Hemolysin activity in the culture supernatant and cell lysate was assayed as previously described (32).

RESULTS

Molecular cloning of the *A. pleuropneumoniae ptxA* gene. Total genomic DNA from *A. pleuropneumoniae* HK361 (serotype 2) was used to construct a library in the lambda replacement vector EMBL3. Approximately 3,000 recombinant plaques were transferred to nitrocellulose and screened for reactivity against convalescent-phase pig serum. Fourteen plaques that reacted with the pig antibody were purified twice, and lysates of each purified clone were screened further by SDS-PAGE and immunoblotting with the same antibody reagent. Four of the lysates from reactive plaques were shown to contain an antigen of 120 kDa (Fig. 1A). The remaining 10 plaque lysates contained reactive antigens of different sizes, including 150, 109, and 63 kDa. Lysates were examined for toxicity against porcine alveolar macrophages. All four lysates containing the 120-kDa antigen were toxic to porcine macrophages in culture. None of the other lysates tested showed any toxicity for porcine macrophages.

The DNA from one of the recombinant bacteriophages that expressed the 120-kDa antigen and cytotoxic activity (recombinant 13) was purified and digested with a variety of restriction enzymes. The insert DNA was shown to be approximately 19 kb in length. No *Sal*I digestion sites were present within the insert DNA. This enzyme was used to remove the bacteriophage DNA for subcloning of the insert into plasmid vector pUC18. This was transformed into *E. coli* JM109. Separate transformants termed JM109(pPTX1) and -(pPTX2), were shown to carry the insert DNA in both possible orientations (data not shown). Both recombinants expressed the 120-kDa antigen, apparently in similar quantity, in the cell fraction (Fig. 1B). Furthermore the 120-kDa polypeptide was detected in the cell-free culture supernatant (Fig. 1B), and the supernatant was toxic to porcine alveolar cells (Fig. 2). The toxic activity was heat labile at 100°C and was neutralized by convalescent-phase pig serum at a dilution of 1:128. The recombinant 120-kDa polypeptide was recognized by rabbit antiserum raised to the native 120-kDa polypeptide purified from the Hly^- mutant of *A. pleuropneumoniae* (HK361e) by preparative SDS-PAGE (Fig. 3). However, no reaction was found with VPG14, a monoclonal antibody directed against the 109-kDa hemolysin of *A. pleuropneumoniae* HK361. Furthermore, no hemolytic activity was present in the culture supernatant or cell lysates from *E. coli*(pPTX1), which contained cytotoxic activity. This confirmed the cloning of the DNA encoding the active cytotoxin of *A. pleuropneumoniae*, recombinant PTX (rPTX), in *E. coli*.

Analysis of the kinetics of appearance of the recombinant 120-kDa antigen revealed that the rPTX was secreted into

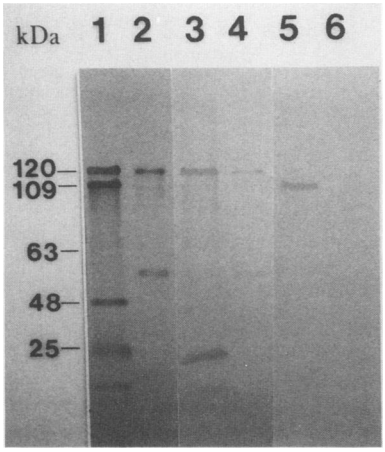

FIG. 3. Comparison of native and recombinant PTX by immunoblot analysis. Lanes: 1, 3, and 5, culture supernatant from *A. pleuropneumoniae* HK361; 2, 4, and 6, culture supernatant from *E. coli* DH1(pPTX1). Lanes 1 and 2 were probed with convalescent-phase pig serum absorbed with *E. coli* as described in Materials and Methods; lanes 3 and 4 were probed with rabbit antiserum raised to PTX purified by preparative gel electrophoresis; and lanes 5 and 6 were probed with monoclonal antibody VPG14, which is specific for the 109-kDa hemolysin.

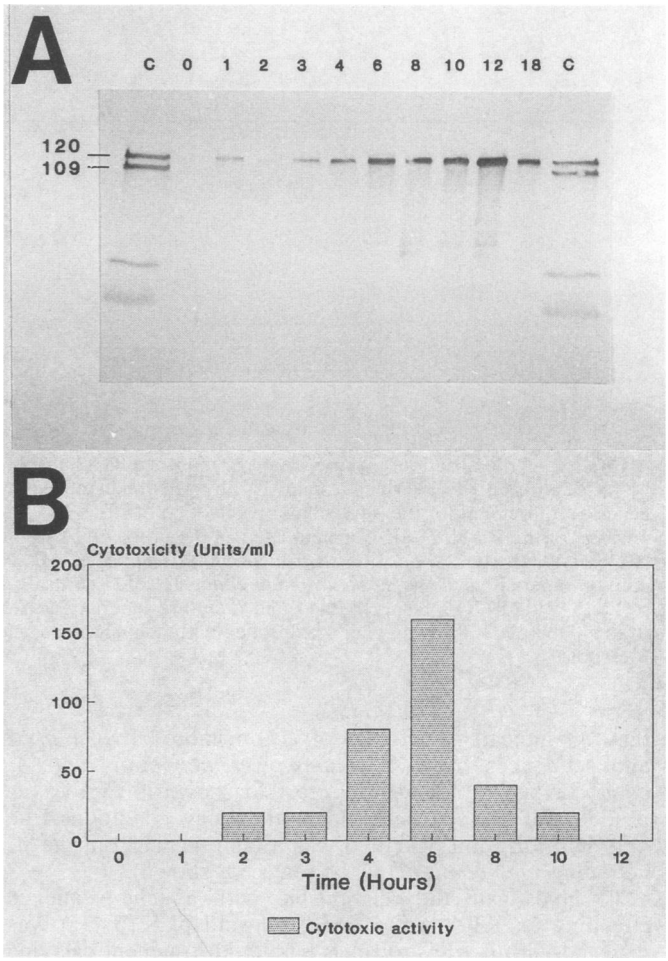

FIG. 4. Kinetics of production of 120-kDa antigen and cytotoxic activity into the culture supernatant from *E. coli* DH1(pPTX1) during growth. (A) Immunoblot analysis of culture supernatants. Lanes c contained control supernatant from *A. pleuropneumoniae* HK361 (8 h); for the other lanes, the time of sampling (in hours) is shown above each lane. Culture supernatants were concentrated by precipitation with 23% ethanol and analyzed by Western blotting with convalescent-phase pig serum absorbed with *E. coli* lysate, as described in Materials and Methods, as a probe. (B) Cytotoxic activity of culture supernatants sampled during growth of *E. coli* DH1(pPTX1). The results are representative of three independent experiments.

and accumulated in the culture supernatant, yielding a maximum of immunoreactive material at approximately 12 h (Fig. 4A). The toxic activity reached a peak after 6 h of incubation and then rapidly declined (Fig. 4B). Toxic activity was not detectable in cultures after 12 h of incubation.

Analysis of pPTX1. Restriction endonuclease analysis of plasmid pPTX1 DNA was used to generate a physical map of the PTX-coding region (Fig. 5A). pPTX1 comprised 18.9 kb of DNA ligated to pUC18. Deletions of pPTX1 were constructed in vitro, transformed into the host strain, and tested for production of the 120-kDa polypeptide (Fig. 5B). The minimal region of DNA essential for the intracellular expression of the 120-kDa polypeptide (calculated to be approximately 2.9 kb) was localized to a region of approximately 4.3 kb between sites for *Xho*I and *Eco*RI cleavage (Fig. 5A). This was confirmed by the construction of pPTX15 with an insert of 4.3 kb that expressed a polypeptide identical in size to the native PTX (Fig. 5B). However, this polypeptide was only found in whole cells and was not present in the cell-free culture supernatant. Further reduction in size of the insert to 3.7 kb (pPTX16) gave no expression of the 120-kDa antigen or cross-reactive material (Fig. 5B). Analysis of cells harboring pPTX10 revealed the production of a truncated polypeptide of approximately 42 kDa (Fig. 5B). We have termed the structural gene *ptxA*.

Prevalence of *ptxA* in *A. pleuropneumoniae*. The distribution of the structural gene *ptxA* was assessed by Southern blot analysis of total genomic DNA from all 13 designated serotype reference strains of *A. pleuropneumoniae*. Genomic DNA was digested with *Pvu*II and probed with radioactively labeled DNA prepared from the internal 1.7-kb *Pvu*II fragment of *ptxA* (Fig. 5A). Strong hybridization was observed between the probe and a 1.7-kb genomic fragment in the donating strain HK361, confirming this organism as the source of the cloned DNA (Fig. 6). Similarly, strong hybridization was found with a 1.7- to 2.0-kb fragment in the reference strains of serotypes 2, 3, 4, 6, and 8 at both low and high stringencies. The hybridizing fragment was slightly larger in the case of the serotype 3, 4, 6, and 8 strains. However, at low stringency, no hybridization between the *ptxA* probe and the genomic DNA from any of the other type strains of *A. pleuropneumoniae* (with the exception of serotype 10 strain 13039, which showed a faint signal in the region of 12 kb) was found (Fig. 6).

The distribution of *ptxA* among other bacteria of the genus *Actinobacillus* was examined. At low stringency, a strong hybridization signal was observed between the probe DNA and large (>12-kb) fragments of *Pvu*II-digested genomic DNA from strains of toxigenic *Actinobacillus actinomycetemcomitans* and *A. equuli* (Fig. 7), indicating the presence of a related sequence with a different genetic structure. A faint signal was detected with DNA from *Actinobacillus lignieresii* and *A. suis* under the same conditions. With the method of Meinkoth and Wahl (21), and taking 41 mol% of G+C as the mean for the genus *Actinobacillus* (20), it was calculated

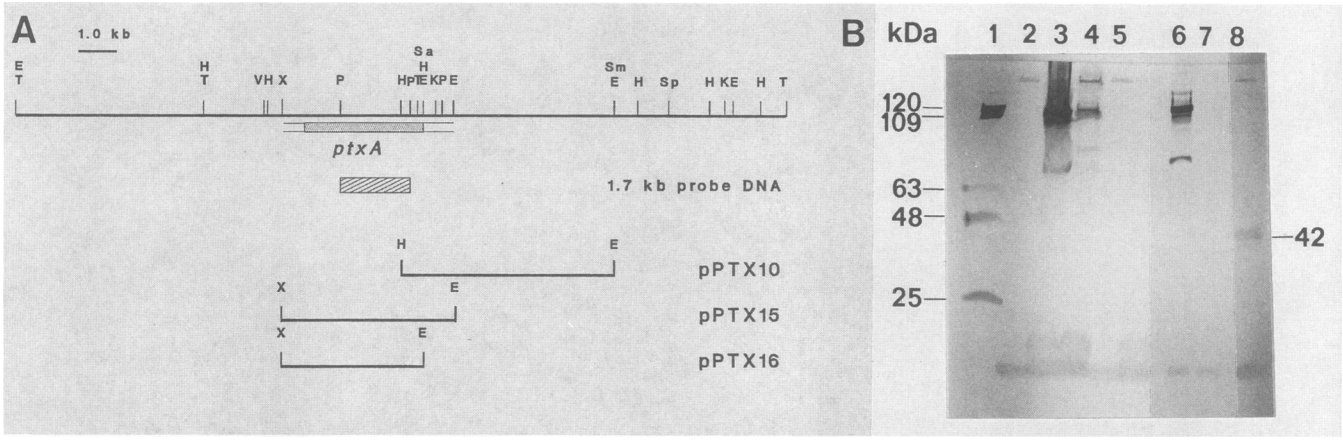

FIG. 5. (A) Restriction enzyme cleavage map of pPTX1 insert DNA (18.9 kb). Inserts from selected recombinant plasmids (pPTX10, pPTX15, and pPTX16), derived from pPTX1 by deletion in vitro, are shown. The approximate position and limits (broken lines) of the minimal genetic requirement for the intracellular production of rPTX (*ptxA*), as defined by analysis of the deletion derivatives, is also given, as is the position of the 1.7-kb *Pvu*II fragment used as the probe DNA in the hybridization experiments. E, *Eco*RI; T, *Pst*I, H, *Hin*dIII; V, *Eco*RV; X, *Xho*I; P, *Pvu*II; Sa, *Sac*I; K, *Kpn*I; Sm, *Sma*I; Sp, *Sph*I. (B) Immunoblot analysis of *E. coli* carrying derivatives of pPTX1. Lanes: 1, culture supernatant from *A. pleuropneumoniae* HK361 (control); 2 through 5, cell lysates from *E. coli* JM109 carrying pUC18, pPTX1, pPTX15, and pPTX16, respectively; 6 and 7, culture supernatants from *E. coli* JM109 carrying pPTX1 and pPTX15, respectively; 8, cell lysate from JM109(pPTX10). The filter was probed with convalescent-phase pig serum absorbed with *E. coli* lysate as described in Materials and Methods.

that this indicated homology of 71% or more between *ptxA* and sequences from *A. actinomycetemcomitans* and *A. equuli*. When a blot identical to that shown in Fig. 7 was probed and washed under high-stringency conditions, the only hybridization observed was a faint signal from the *A. actinomycetemcomitans* DNA (data not shown).

To investigate the relationship with a gene sequence encoding an RTX group toxin, plasmid pLKT5 (35) was digested with *Eco*RV to release a 3.1-kb fragment carrying the *lktCA* genes and probed with the 1.7-kb *Pvu*II *ptxA* gene probe. Very faint hybridization was detected with the purified plasmid DNA (Fig. 8). Similarly, a faint hybridization signal was found between the *ptxA* probe and plasmid pAPH1, which carries the genetic elements required for the expression of the hemolysin (HlyII) isolated from *A. pleuropneumoniae* HK361 (serotype 2) (unpublished data).

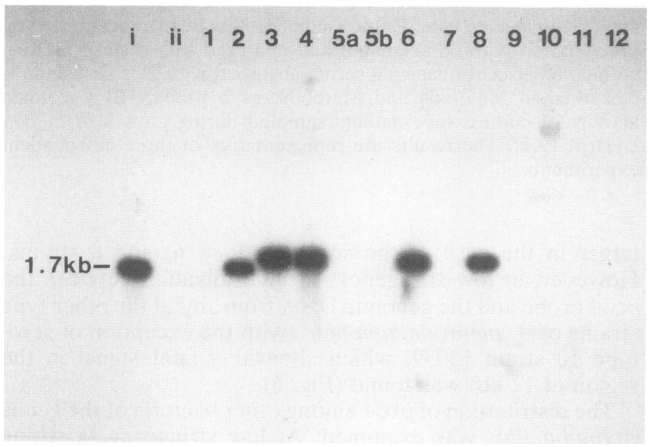

FIG. 6. Detection of *ptxA* among the 13 serotype reference strains of *A. pleuropneumoniae*. Southern hybridization analysis was performed on *Pvu*II-digested total genomic DNA (1.0 μg per lane). The filter was probed with the 1.7-kb *Pvu*II DNA fragment from the center of the *ptxA* gene. Lanes: i and 1 through 12, *A. pleuropneumoniae* strains; i, HK361; ii, *E. coli* JM109 as a negative control; 1, 4074; 2, S1536; 3, S1421; 4, M62; 5a, K17; 5b, L20; 6, Femø; 7, WF83; 8, 405; 9, CVI13261; 10, 13039; 11, 56153; 12, 8329. The filter was hybridized and washed under low-stringency conditions as described in Materials and Methods.

DISCUSSION

The gene determining the extracellular, heat-labile cytotoxin of *A. pleuropneumoniae* serotype 2 was cloned in *E. coli*. The 120-kDa antigen, initially expressed from a lambda EMBL3 library, was cytotoxic to porcine alveolar cells, demonstrating that the genetic determinants for active cytotoxin had been isolated. When subcloned into pUC18 and transformed into *E. coli*, the resulting recombinant plasmid expressed the 120-kDa polypeptide. Furthermore, the active toxin was exported from *E. coli* into the culture supernatant,

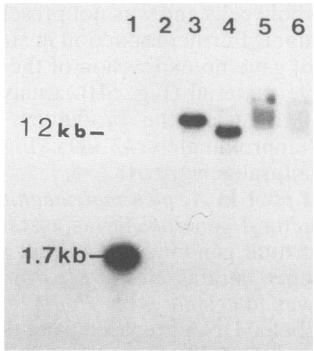

FIG. 7. Southern hybridization analysis of DNA from *Actinobacillus* spp. probed with *ptxA*-derived 1.7-kb *Pvu*II probe DNA. Lanes: 1, *A. pleuropneumoniae* HK361; 2, *E. coli* JM109; 3, *A. actinomycetemcomitans* JP2; 4, *A. equuli*; 5, *A. lignieresii*; 6, *A. suis*. A sample of 1.0 μg of DNA digested with *Pvu*II was applied to each lane. The filter was hybridized and washed under low-stringency conditions as described in Materials and Methods.

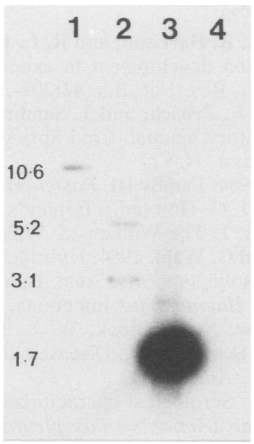

FIG. 8. Comparison of *ptxA* with DNA from the cloned leukotoxin gene of *P. haemolytica* and the hemolysin (HlyII) gene of *A. pleuropneumoniae* serotype 2 by Southern hybridization. The filter was probed with the 1.7-kb *Pvu*II *ptxA* gene probe of pPTX1. Lanes: 1, pAPH1 digested with *Kpn*I to release the fragment carrying the structural gene (10.6 kb); 2, pLKT5 digested with *Eco*RV; 3, pPTX1 digested with *Pvu*II; 4, pUC18 digested with *Eco*RI. A sample of 0.05 μg of DNA was applied to each lane. Filters were hybridized at 55°C and washed as described in Materials and Methods.

suggesting that all of the genetic requirements for the production and secretion of active toxin (rPTX) had been cloned and were freely expressed in *E. coli*. The 120-kDa recombinant protein was not distinguishable from native PTX on the criteria of its size, reaction with specific antibody, heat lability, and specificity for porcine alveolar cells.

Recombinant toxin activity was expressed in the exponential phase of growth. However, the toxin activity declined rapidly after the peak of expression, suggesting that the toxin was unstable in the culture conditions. Furthermore, since the immunoreactive 120-kDa antigen was maximal at 12 h, but no toxicity remained at this time, it appears that production of the toxin had ceased by 12 h and that degradation of the 120-kDa polypeptide was not the reason for the decline of toxin activity.

Deletion mutagenesis was used to localize the structural gene for the toxin to a 4.3-kb fragment near the center of the insert DNA. Since the structural gene lies within a large region of *A. pleuropneumoniae* DNA and expression levels were similar in both orientations relative to the vector, it is likely that the gene is expressed in *E. coli* from an *A. pleuropneumoniae* promoter. Deletion of pPTX1 to yield pPTX15 also suggested that one or more regions of the cloned DNA are involved in export of rPTX into the culture supernatant. Our results therefore imply that, like the *E. coli hlyCABD* cluster (38) and the *Pasteurella haemolytica lktCABD* cluster (35), the export genes for pleurotoxin may be linked to the toxin gene *ptxA*. This is in contrast to the hemolysin II genes of *A. pleuropneumoniae*, in which intact *appBD* genes are located at a different site on the chromosome from *appCA* (5).

It has been suggested that the hemolysin and cytotoxin of serotype 2 *A. pleuropneumoniae* strains are related, i.e., that the 105-kDa hemolysin is an inactive form of the 120-kDa cytotoxin (13). In some immunoblots of rPTX probed with convalescent-phase pig serum we observed a polypeptide of approximately 109 kDa (the expected position of the hemolysin protein). However, our results do not support the idea that this represents the hemolysin. First, this band did not react in immunoblot experiments with either anti-PTX polyclonal antiserum or with a monoclonal antibody (VPG14) that recognizes the 109-kDa hemolysin band. Second, the gene encoding the 109-kDa hemolysin of serotype 2 was separately cloned from the same DNA library to yield plasmid pAPH1 (data not shown), and its restriction map was found to resemble the published restriction map of the hemolysin gene *appA* from *A. pleuropneumoniae* serotype 5 (4). It bore no similarities to the map of the *ptx* DNA insert. Third, no hemolytic activity was detected in the cytotoxin preparations from *E. coli* pPTX1. Finally, a probe of the central *Pvu*II fragment of *ptxA* hybridized only very weakly to pAPH1, indicating that these two molecules were encoded by distinct sequences of DNA.

We detected the gene for pleurotoxin in only 5 of the 13 serotype reference strains. This was an unexpected finding in view of the widespread detection of the 120-kDa polypeptide in field strains of *A. pleuropneumoniae* (32) and the presence of cytotoxin in cultures of these reference strains with the exception of serotype 6 (15). Clearly, the *ptxA* gene is not universal among *A. pleuropneumoniae* isolates, implying that some other factor, perhaps hemolysin protein or a different cytotoxin, confers the cytotoxic activity observed in strains lacking *ptx*. Kamp et al. have recently distinguished three groups of cytotoxin on the basis of studies with monoclonal antibodies (12). One of the groups of cytotoxins proposed by these authors, a 120-kDa protein, was found in serotypes 2, 3, 4, and 8. Since a sequence hybridizing strongly with the *ptxA* probe was present in all of these reference strains, it is likely that the cytotoxins from these organisms represent a distinct group from those of other serotypes. We detected strong hybridization with the *ptxA* probe in the DNA of serotype 6 strain Femø. In view of the findings of Kamp et al. (12), it is likely that this represents a pseudogene or that its product is expressed intracellularly but not secreted.

We detected no hybridization between the gene probe for *ptxA* and the DNA of *A. pleuropneumoniae* serotype 1 strain 4074 (Fig. 6). Also, the published map of the gene for HlyI of this strain (*hlyIA* [10]) was different from that of the *ptx* region. Since *hlyIA* and the gene for HlyII (*appA*) are different from *ptxA* and from each other, at least three genetically diverse cytolytic proteins are produced by this species of bacteria. Both the HlyI and HlyII proteins belong to the RTX family of bacterial toxins (35). Given that there is detectable sequence homology between *ptxA* and both *lktCA* on pLKT5 and the gene for HlyII on pAPH1 (Fig. 8), it is probable that PTX is also related to the RTX group.

Furthermore, although the work of Kamp et al. (12, 15) suggests that the production and type of cytolysins may be related to serotype, there are clearly exceptions to this general rule. Among the field isolates of *A. pleuropneumoniae* examined in this laboratory, we detected the *ptxA* gene in a strain belonging to serotype 5 (data not given).

In view of the common features of the pathogenesis of contagious pleuropneumonia, the absence of a *ptxA* gene in some reference strains suggests that the 120-kDa cytotoxin is not essential for the pathogenicity of this organism. A recent study has shown that, in serotype 2, the Ca^{2+}-dependent hemolysin (HlyII) is not essential for lethality or for the formation of the typical lesions of pleuropneumonia (33). It has also been shown that the hemolysins I and II have cytotoxic activity in addition to hemolytic properties (12). It is therefore possible that the different toxin proteins, with distinct genetic determinants, may substitute for each other in producing the same disease syndrome. The alternative

conclusion is that another, as yet unrecognized, virulence determinant is essential for the pathogenicity of *A. pleuropneumoniae*.

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REFERENCES

1. **Bendixen, P. H., P. E. Shewen, S. Rosendal, and B. N. Wilkie.** 1981. Toxicity of *Haemophilus pleuropneumoniae* for porcine lung macrophages, peripheral blood monocytes, and testicular cells. Infect. Immun. **33**:673–676.
2. **Bertram, T.** 1988. Pathobiology of acute pulmonary lesions in swine infected with *Haemophilus* (*Actinobacillus*) *pleuropneumoniae*. Can. Vet. J. **29**:574–577.
3. **Bertram, T. A.** 1990. *Actinobacillus pleuropneumoniae*: molecular aspects of virulence and pulmonary injury. Can. J. Vet. Res. Suppl. **54**:S53–S56.
4. **Chang, Y.-F., R. Young, and D. K. Struck.** 1989. Cloning and characterization of a hemolysin gene from *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. DNA **8**:635–647.
5. **Chang, Y.-F., R. Young, and D. K. Struck.** 1991. The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. J. Bacteriol. **173**:5151–5158.
6. **Clewell, D. B., and D. J. Helinski.** 1970. Properties of a supercoiled DNA-protein relaxation complex and strand specificity of the relaxation event. Biochemistry **9**:4428–4440.
7. **Devenish, J., and S. Rosendal.** 1989. Identification of the heat-labile hemolysin of *Actinobacillus pleuropneumoniae* serotype 1. Can. J. Vet. Res. **53**:251–254.
8. **Frey, J., J.-B. Deillon, D. Gygi, and J. Nicolet.** 1991. Identification and partial characterization of the hemolysin (HlyII) of *Actinobacillus pleuropneumoniae* serotype 2. Vet. Microbiol. **28**:303–312.
9. **Frey, J., and J. Nicolet.** 1988. Purification and partial characterization of a hemolysin produced by *Actinobacillus pleuropneumoniae* type strain 4074. FEMS Microbiol. Lett. **55**:41–46.
10. **Gygi, D., J. Nicolet, J. Frey, M. Cross, V. Koronakis, and C. Hughes.** 1990. Isolation of the *Actinobacillus pleuropneumoniae* haemolysin gene and the activation and secretion of the prohaemolysin by the HlyC, HlyB and HlyD proteins of *Escherichia coli*. Mol. Microbiol. **4**:123–128.
11. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
12. **Kamp, E. M., J. K. Popma, J. Anakotta, and M. A. Smits.** 1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. Infect. Immun. **59**:3079–3085.
13. **Kamp, E. M., J. K. Popma, and M. A. Smits.** 1990. Proceedings of the 11th International Pig Veterinary Society Congress, Lausanne, Switzerland, p. 20.
14. **Kamp, E. M., J. K. Popma, and L. A. M. G. Van Leengoed.** 1987. Serotyping of *Haemophilus pleuropneumoniae* in the Netherlands: with emphasis on heterogeneity within serotype 1 and (proposed) serotype 9. Vet. Microbiol. **13**:249–257.
15. **Kamp, E. M., and L. A. M. G. Van Leengoed.** 1989. Serotype-related differences in production and type of heat-labile hemolysin and heat-labile cytotoxin of *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. J. Clin. Microbiol. **27**:1187–1191.
16. **Kilian, M., J. Nicolet, and E. L. Biberstein.** 1978. Biochemical and serological characterization of *Haemophilus pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964 and proposal of a neotype strain. Int. J. Syst. Bacteriol. **28**:20–26.
17. **Kume, K., T. Nakai, and A. Sawata.** 1986. Interaction between heat-stable hemolytic substance from *Haemophilus pleuropneumoniae* and porcine pulmonary macrophages in vitro. Infect. Immun. **51**:563–570.
18. **Liggett, A. D., L. R. Harrison, and R. L. Farrell.** 1987. Sequential study of lesion development in experimental haemophilus pleuropneumonia. Res. Vet. Sci. **42**:204–221.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mannheim, W.** 1984. Family III. *Pasteurellaceae*, p. 550–575. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
21. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138**:267–284.
22. **Nicolet, J.** 1986. *Haemophilus* infections, p. 426–436. *In* A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine. Iowa State University Press, Ames.
23. **Nielsen, R.** 1985. Serological characterization of *Haemophilus pleuropneumoniae* (*Actinobacillus pleuropneumoniae*) strains and proposal of a new serotype: serotype 9. Acta Vet. Scand. **26**:501–512.
24. **Nielsen, R.** 1985. Serological characterization of *Haemophilus pleuropneumoniae* (*Actinobacillus pleuropneumoniae*) strains and proposal of a new serotype: serotype 10. Acta Vet. Scand. **26**:581–586.
25. **Nielsen, R.** 1986. Serology of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* serotype 5 strains: establishment of subtypes A and B. Acta Vet. Scand. **27**:49–58.
26. **Nielsen, R.** 1986. Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. **27**:453–455.
27. **Nielsen, R., and P. J. O'Connor.** 1984. Serological characterization of 8 *Haemophilus pleuropneumoniae* strains and proposal of a new serotype: serotype 8. Acta Vet. Scand. **25**:96–106.
28. **Rosendal, S., and D. A. Boyd.** 1982. *Haemophilus pleuropneumoniae* serotyping. J. Clin. Microbiol. **16**:840–843.
29. **Rosendal, S., J. Devenish, J. I. MacInnes, J. H. Lumsden, S. Watson, and H. Xun.** 1988. Evaluation of heat-sensitive, neutrophil toxic, and hemolytic activity of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae*. Am. J. Vet. Res. **49**:1053–1058.
30. **Rosendal, S., W. R. Mitchell, M. Weber, M. R. Wilson, and M. R. Zaman.** 1980. Proceedings of the 6th International Pig Veterinary Society Congress, Copenhagen, Denmark, p. 221.
31. **Rycroft, A. N., and J. M. Cullen.** 1990. Complement resistance in *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* infection of swine. Am. J. Vet. Res. **51**:1449–1453.
32. **Rycroft, A. N., D. Williams, J. M. Cullen, and J. Macdonald.** 1991. The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. J. Gen. Microbiol. **137**:561–568.
33. **Rycroft, A. N., D. Williams, I. A. P. McCandlish, and D. J. Taylor.** 1991. Experimental reproduction of acute lesions of porcine pleuropneumonia with a haemolysin-deficient mutant of *Actinobacillus pleuropneumoniae*. Vet. Rec. **129**:441–443.
34. **Sebunya, T. N., and J. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331–1337.
35. **Strathdee, C. A., and R. Y. C. Lo.** 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* leukotoxin determinant. J. Bacteriol. **171**:916–928.
36. **Tsai, C.-C., B. J. Shenker, J. N. DiRienzo, D. Milamud, and N. S. Taichman.** 1984. Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. Infect. Immun. **43**:700–705.
37. **Van Leengoed, L. A., E. M. Kamp, and J. M. A. Pol.** 1989. Toxicity of *Haemophilus pleuropneumoniae* to porcine lung macrophages. Vet. Microbiol. **19**:337–349.
38. **Welch, R. A., and S. Pellett.** 1988. Transcriptional organization of the *Escherichia coli* hemolysin genes. J. Bacteriol. **170**:1622–1630.
39. **Yanisch-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–199.